University of Alberta
Printing Department

THE UNIVERSITY OF ALBERTA

THE CHEMICAL COMPOSITION OF MILK FAT FRACTIONS

by

PI-CHEN CHEN

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF DAIRY AND FOOD SCIENCE

EDMONTON, ALBERTA

DECEMBER, 1965

UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled

THE CHEMICAL COMPOSITION OF

MILK FAT FRACTIONS

submitted by Pi-chen Chen in partial fulfilment of the requirements for the degree of Master of Science.

ABSTRACT

Eight fractions of milk fat were obtained by fractional crystallization from acetone. A specially designed instrument is described for the separation of fat crystals at controlled temperatures in a thermostat bath. It was found that both trisaturated glycerides and unsaturated triglycerides were present in all of the separated fractions.

The fatty acid composition of the fractions was determined by Gas-Liquid Chromatography (GLC). The higher melting fractions were characterized by being low in short chain fatty acids and high in saturated fatty acids. The intermediate fractions were similar in composition to the original fat. The lower melting fractions were high in both short chain and unsaturated fatty acids. The trisaturated glyceride contents of the July milk fat and its fractions were determined. The fatty acid composition of all of the trisaturated glycerides were also determined by GLC. Short chain fatty acids were combined with palmitic or stearic acid in the high melting glycerides and with unsaturated acids in the low melting glycerides. Oleic acid was combined with myristic, palmitic or stearic acid in the unsaturated triglycerides. Myristic acid seemed to be combined with both palmitic and stearic acids to form the high melting glyceride.

From a consideration of the trisaturated glyceride contents of July fat and its fractions, the distribution pattern of the fatty

acids among the glycerides of milk fat and its fractions were assumed to be nonrandom.

The distribution of partial glycerides and cholesterol was determined. Partial glycerides tended to accumulate in the lower melting fractions. Cholesterol accumulated largely in the residual fraction.

ACKNOWLEDGEMENTS

The writer wishes to express sincere appreciation to her professors and fellow students who have been so kind as to discuss academic problems with her during her study at the Department of Dairy and Food Science, University of Alberta. Her deepest gratitude, however, is to Dr. J.M. deMan, under whose direction this work was carried on, and whose guidance and criticism have been of the utmost value. Others who assisted her in a variety of ways were the non-academic staff members of the Department. To these individuals also she is very thankful. Finally, she likes to thank her husband, Mr. C.C. Chen, for his endless encouragement.

TABLE OF CONTENTS

INTRODUCTION 1

REVIEW OF LITERATURE 3

I. CHEMISTRY OF THE TRIGLYCERIDES 3

A. Fatty Acid Composition of Milk Fat 3

1. Saturated fatty acids 3

2. Unsaturated fatty acids 6

B. Origin of Fatty Acids 8

C. The Distribution Pattern of Fatty Acids in the Glycerides of Milk Fat 12

II. PARTIAL GLYCERIDES 14

III. CHOLESTEROL 15

IV. METHODS FOR THE FRACTIONATION OF FATS 16

A. Fractional Crystallization 16

B. Countercurrent Distribution 18

C. Thermal Gradient Fractionation 18

D. Thin-Layer Chromatography 19

EXPERIMENTAL METHODS 20

I. PREPARATION OF MILK FAT 20

II. FRACTIONAL CRYSTALLIZATION 20

III. DETERMINATION OF THE FATTY ACID COMPOSITION 23

IV. SEPARATION OF GLYCERIDES BY CHROMATOGRAPHY ON FLORISIL 23

V. CHOLESTEROL DETERMINATION 24

VI. TRISATURATED GLYCERIDE DETERMINATION 26

RESULTS 27

DISCUSSION 42

I. FRACTIONAL CRYSTALLIZATION FROM SOLVENT 42

II. THE COMPONENT GLYCERIDES 43

III. PARTIAL GLYCERIDES AND CHOLESTEROL 46

IV. MINOR CONSTITUTIONS IN SOME OF THE LOWER MELTING FRACTIONS 47

BIBLIOGRAPHY 48

LIST OF FIGURES AND TABLES

Fig. 1. Schematic representation of the filtering apparatus used for the crystallization and filtration of milk fat fractions at controlled temperatures 22

Table 1. Fatty acid composition of milk fat determined by ester fractionation analysis (Mole %) 4

Table 2. Yield (in weight %) of fractions obtained by fractional crystallization of milk fat from acetone 31

Table 3. Component acids (in weight %) in the July milk fat and the fractions obtained from it by fractional crystallization from acetone 32

Table 4. Component acids (in weight %) in the December milk fat and the fractions obtained from it by fractional crystallization from acetone 33

Table 5. Component acids (in weight %) in the trisaturated glycerides (GS_3) of July milk fat and of fractions obtained from it by fractional crystallization from acetone 34

Table 6. Fatty acid distribution in the +15°C fraction and in the trisaturated glycerides and the unsaturated triglycerides obtained from it 35

Table 7. Fatty acid distribution in the +5°C fraction and in the trisaturated glycerides and the unsaturated triglycerides obtained from it 36

Table 8. Fatty acid distribution in the residual fraction and in the trisaturated glycerides and the unsaturated triglycerides obtained from it 37

Table 9. Content of trisaturated glycerides in the July milk fat and the fractions obtained from it by fractional crystallization from acetone 38

Table 10. Ratios of weight % of the major fatty acids in the trisaturated glycerides of July milk fat and its fractions to that of the fatty acids in the original milk fat and its fractions 39

Table 11. Mono-, di-, and tri-glyceride contents (in weight %) of July and December milk fats and the fractions obtained from them by fractional crystallization from acetone 40

Table 12. Free, ester, and total cholesterol contents of July and December milk fats and the fractions obtained from them by fractional crystallization from acetone 41

THE CHEMICAL COMPOSITION OF MILK FAT FRACTIONS

INTRODUCTION

Much less is known about milk fat glycerides than about the fatty acid composition of milk fat. The composition of the individual glycerides will depend on the number of fatty acids available for glyceride formation. From the very large number of fatty acids identified in milk fat, the possible number of different mixed glycerides that may exist is legion which indicates that any detailed study of milk fat triglycerides is a difficult problem. Generally the properties of a fat or oil depend on the component fatty acids, but the composition of the component glycerides also plays an important role. The purpose of the present work has been to separate milk fat into less complex fractions for further study.

Fractional crystallization from a solvent was chosen for the present study. It has been employed for the determination of the glyceride composition of a number of fats, especially by Hilditch (54). This technique is easy to perform and the molecules are not changed by the process. Although this method does not permit complete separation of a fat into individual glycerides, the fractions separated have simpler glyceride compositions which can be more easily determined from their fatty acid composition.

Milk fat is composed of triglycerides in which are dissolved free fatty acids, mono- and diglycerides, and a heterogenous group of materials called the unsaponifiable matter. In milk fat the unsaponifiable matter is largely cholesterol. In this study cholesterol content and the mono-, di-, and tri-glyceride content of July and December milk fats and their fractions obtained by fractional crystallization from acetone were analyzed.

REVIEW OF LITERATURE

I. CHEMISTRY OF THE TRIGLYCERIDES

It has been recognized since the work of Chevreul (17) that natural fats are glycerol esters of fatty acids. The chemistry of the triglycerides is determined by the component fatty acids, their configuration, and their distribution pattern in the glyceride molecules.

A. Fatty Acid Composition of Milk Fat

The triglycerides of milk fat probably contain a greater variety of fatty acids than any other natural fat. Table 1 presents quantitative data for the major component fatty acids of milk fat as determined by Hilditch and his co-workers who employed the techniques of ester distillation and alkali isomerization (51, 52). From these investigations one of the most striking features to emerge was that milk fat differed from the depot fat in that it contained significant amounts of steam-volatile components, mainly butyric, caproic, caprylic and capric acid.

1. Saturated fatty acids

The results of ester-fractionation analysis depended on the fundamental assumption that the acids present represented those of a homologous series of saturated and unsaturated n-straight-chain components, each possessing an even number of carbon atoms; the composition of each ester fraction was determined from its saponification equivalent, its iodine value and its behaviour on alkali

Table 1. Fatty acid composition of milk fat determined by ester fractionation analysis.* (Mole%)

Saturated		
Butyric	8.1	10.5
Caproic	2.8	4.6
Caprylic	2.5	1.3
Capric	3.7	2.7
Lauric	4.4	2.6
Myristic	12.5	9.6
Palmitic	23.2	23.4
Stearic	7.6	9.7
Chain-length $>C_{18}$	1.0	0.6
Unsaturated		
Decenoic	0.4	0.3
Dodecenoic	0.9	0.2
Tetradecenoic	1.7	1.0
Hexadecenoic	3.9	2.1
Octadecenoic	24.8	28.6
Octadecadienoic	2.9	1.8
Chain-length $>C_{18}$	0.2	1.0
*Reference	(51)	(52)

isomerization. For a long time, many saturated acids, which it was deduced were present, were not formally identified, although for most components a melting point, saponification equivalent, iodine value, and combustion analysis were recorded (29). As milk fat is now known to contain iso and anteiso acids as well as normal odd numbered acids, identification of individual fractions demands more conclusive proof than was the case before the occurrence of these trace constituents was established. However, the saturated components listed in Table 1 were basically established. In addition to the series of even numbered saturated n-fatty acids listed in Table 1. Hansen, Shorland, and Cooke (42) have recently reported the isolation of trace amounts of several high molecular weight saturated n-acids in milk fat, namely, eicosanoic (arachidic) acid, docosanoic (behenic) acid, tetracosanoic (lignoceric) acid, and hexacosanoic (cerotic) acid.

Natural fats are no longer regarded as containing only even number saturated and unsaturated straight chain fatty acids. Shorland and his co-workers isolated trace amounts of the saturated branched-chain fatty acids with both odd and even numbers of carbon atoms and n-odd number fatty acids from milk fat (37, 38, 39, 40, 41, 42, 100, 101).

Recent studies using Gas Liquid Chromatography (GLC) Patton et al. (86) and Herb and co-workers (47, 75) have confirmed the presence and proportions of the main component saturated acids and have added to the list of minor components. In one of the most

recent studies, Magidman et al. (75) identified at least 60 fatty acids, including several not previously reported, such as odd numbered carbon chain length monoethenoid acids from C_{15} to C_{23}.

2. Unsaturated fatty acids

No unsaturated acid having less than ten carbon atoms has been found in milk fat, though as early as 1912 Smedley (102) deduced the presence of unsaturated acids of chain length less than 18 carbon atoms. Other workers (11, 34, 35, 50) substantiated Smedley's postulation for the presence of decenoic acid and found evidence of C_{12}, C_{14}, and C_{16} monoethenoid acids. The double bond was claimed to be in the 9, 10 position relative to the carboxyl group (51). An odd numbered unsaturated acid (heptadec-cis-9-enoic acid) has been identified (43), and GLC evidence (47) suggests that others are present in trace amounts.

For a long time it was considered that the C_{18} monoethenoid component consisted entirely of oleic acid. In 1928, Bertram's (6) discovery of vaccenic acid (trans-11-octadecenoic) led to further studies by others (19, 89) who also concluded that octadecenoic acids contained positional as well as geometric isomers. Until recently, the lower unsaturated acids were thought to be exclusively cis forms. However, in 1954, Smith and co-workers (103) presented evidence of trans components in C_{12}, C_{14}, C_{16}, and C_{18} monoethenoid fatty acid fractions. In their detailed study of cow milk fat, Backderf and Brown (3) deduced the presence of octadec-trans-16-enoic acid. The unequivocal identification of octadec-

trans-16-enoic acid was achieved very recently by Hansen and Cooke (44).

The diethenoid C_{18} acids of milk fat have been the subject of much discussion. In 1949. White and Brown (108) claimed to have isolated tetra-bromolinoleic acid from butter fat in amounts corresponding to about 70% of the octadecadienoic acid. In a recent paper, Sambasivarao and Brown (93) presented conclusive evidence for the presence of a similarly high proportion of linoleic acid, the remainder consisting of positional isomers having widely separated double bonds with cis-trans configuration.

Another consideration regarding the octadecadienoic acids is whether the double bonds are separated by three carbon atoms or whether they are conjugated. Using spectrophotometric analysis, Scott et al. (98) confirmed the presence of conjugated and non-conjugated dienes. The nonconjugated dienoic acids were found to be a mixture of cis-cis and either cis-trans or trans-trans isomers. Conjugated dienoic acids were identified as cis-trans and trans-trans isomers.

By the use of alkali-isomerization, Shorland (99) has definitely shown that the C_{18} trienoic acid in New Zealand butter fat consists almost entirely of linolenic acid (octadeca-cis-9, cis-12, cis-15-trienoic acid).

By alkali isomerization, unsaturated acids of longer chain length containing 4, 5 and possibly 6 double bonds have been shown to be present in the milk fat. The occurrence of a C_{20}, a C_{22}, or

a C_{24} dienoic acid was found in milk fat by Bosworth and Brown (11). Later arachidonic acid was isolated from milk fat as its octabromide by Bosworth and Sisson (12). The tetra- and penta-unsaturated acids have the all-*cis* configuration (98). The recent GLC analyses by Herb *et al*. (47) indicated the presence of C_{20} and C_{22} mono-, di-, tri-, tetra-, and penta-unsaturated fatty acids.

B. Origin of Fatty Acids

Before the ability of mammary tissue to synthesize fatty acids was known, it was considered that milk glycerides represented plasma glycerides absorbed by the gland and secreted in a modified form, since the glycerides of milk are unusual in that they contain lower volatile acids which are not present in blood lipids in esterified form. It is now generally accepted that the fatty acids of milk glycerides arise from two distinct sources —— from blood plasma lipids and by direct synthesis in the mammary gland from non-lipid precursors.

The fatty acids of blood lipids can be incorporated into milk lipids. This was demonstrated by feeding fatty acids, which were not normally found in milk fat, to lactating cows and subsequently these acids were detected in the milk fat. From a study of the composition of milk fat of cows given cod-liver oil, Hilditch and Thompson (49) found that increased proportions of highly unsaturated fatty acids, typical of fish oils, appeared in the milk. At about the same time, arteriovenous studies established that blood glycerides were removed from the blood plasma flowing through the

udder of the lactating gland in quantities sufficient to produce milk fat (31). It was observed by Aylward et al. (2) that iodized fat fed to lactating cows was transported in the blood either as glycerides or as sterol esters. Since cholesterol esters of bovine plasma make up nearly 80% of the total lipids, they must be considered as possible precursors of milk fat. Cholesterol esters typically contain large amounts of polyunsaturated fatty acids (linoleic, linolenic and arachidonic acids). This observation led Lough and Garton (73) to suggest that dietary polyunsaturated fatty acids which escape hydrogenation in the rumen are esterified with cholesterol during or after their absorption from the gut. There is some disagreement whether phospholipids or cholesterol esters or both are used for fat synthesis. Meigs et al. (81) suggested that milk fat was derived from blood phospholipids. This idea seems to be supported by Riis et al. (92). These investigators infused plasma containing P^{32}- and C^{14}- labeled lipids into a lactating cow, the plasma having been obtained from a donor (non-lactating) cow to which P^{32}- labeled phosphate and acetate-1-C^{14} had been administered. From a consideration of the resultant specific radioactivities of the lipids of the plasma and milk of the animal, it was calculated that about 50% of the fatty acids of the milk lipids were derived from plasma lipids and that, in addition to triglycerides, cholesterol esters and phospholipids were involved in the transport of fatty acids to the mammary tissue. But arteriovenous difference studies have failed to show a significant uptake of either cholesterol esters or phospholipids by the mammary gland of dairy cattle.

Glascock et al. (30) fed tritium-labeled stearic acid, either as free acid or as triglyceride, to lactating goats and to a cow and subsequently found that as much as 60% of the administered radioactivity appeared in the milk. In the glyceride fatty acids the highest specific activities were observed in both saturated and unsaturated long-chain fatty acids, whereas the radioactivity of the short-chain acids was relatively low. In another perfusion experiment, Lauryssens et al. (70) found that plasma free fatty acids (FFA), which represent a quantitatively minor proportion of plasma lipids, can be rapidly taken up and incorporated into udder glycerides. Following the addition, as the albumin complex, of stearate-1-C^{14} to the perfusing blood, the gland glycerides contained significant amounts of labeled oleic acid in addition to stearic acid. Direct desaturation of stearic acid to give oleic acid apparently occurred; the significance of this process as a source of unsaturated acids for milk fat and the possible quantitative importance of FFA as precursors of milk lipids would seem to merit further investigation in the living animal. Further, Luick and Lucas (74) found that when free C^{14}-labeled stearate was similarly infused into a cow's udder, it was incorporated into milk triglycerides. The incorporation of C^{14}-stearate into milk fat following its infusion into mammary tissue suggests that the plasma lipids are hydrolyzed in the udder and thereby provide a pool of fatty acids for milk fat synthesis. However, the determination of which lipid classes of blood contribute to the mammary gland fatty acid pool remains as a highly important unsolved problem.

The short-chain acids of milk fat were found to arise in the mammary tissue by synthesis from acetate; each of the saturated acids up to and including palmitic acid can be synthesized in this way. Popjak et al. (91) injected intravenously labeled acetate into a lactating goat. Milk samples were collected at intervals after the injection and the specific radioactivities of the component fatty acids were measured. The results showed that synthesis from acetate was rapid and that stearic and oleic almost certainly did not arise in that way, but were derived mainly from the blood. It was suggested by Popjak et al. (91) that although acetate was a major source of carbon for milk fatty acid synthesis, another C_4 compound, derived from the blood, possibly β-hydroxybutyric acid must also be involved. Kumar et al. (69) reported that the carboxyl carbon of C^{14}- labeled β-hydroxybutyrate was incorporated appreciably into milk fat by the perfused lactating bovine udder. The incorporation of the label into the short chain fatty acids, butyric acid, caproic acid, caprylic acid, and capric acid, was especially high. Negligible incorporation was found in the C_{12} and higher acids. Fatty acids synthesis from labeled acetate in the lactating mammary gland of the cow has been described by Kleiber et al. (63).

McCarthy et al. (77) proposed that two mechanisms might be involved in milk fat synthesis: alteration of pre-existing triglyceride molecules and supplemental synthesis. But what proportion of the total fatty acids is synthesized from acetate and β-hydroxybutyrate and what proportion comes from plasma lipids

remains unanswered.

C. The Distribution Pattern of Fatty Acids in the Glycerides of Milk Fat

Fats are not mixtures of simple triglycerides but rather contain mixtures of mixed triglycerides. In 1927 Hilditch and Lea (48) developed an oxidation procedure by which the fully saturated glycerides could be separated from those containing unsaturated components. By using the techniques of fractional crystallization and oxidation it has been possible since that time to separate the glycerides into four categories, depending on the number of saturated fatty acids in the molecule. These have been characterized as GS_3, GS_2U, GSU_2, and GU_3 —— where G represents the glyceride component, S the saturated fatty acids present, and U the unsaturated fatty acids present in the glyceride molecules. In 1952 Greenbank (32, 33) fractionated milk fat in absolute alcohol and studied the glyceride structure of the different fractions. The results showed that some trisaturated but no triunsaturated glycerides were present. He concluded that most of the glycerides were the monosaturated and disaturated type.

Hilditch (54) has proposed a general distribution pattern for the fatty acids in natural fats. In this pattern fatty acids follow a "rule of even distribution". According to this rule,the component fatty acids in a natural fat tend to be distributed as widely as possible among all the triglyceride molecules. According to Longenecker (72), the fatty acids are randomly

distributed. Procedures included fractional crystallization from light petroleum between 7°C and -53°C and oxidative removal of unsaturated acids using $KMnO_4$ in acetone. Jack *et al.* (46, 56) separated milk fat into five fractions and determined the component fatty acids and the fatty acid composition of the fully saturated glycerides in each fraction. Their results conformed closely to the pattern predicted by even distribution. Kartha (62) showed data for cow milk fat that he claimed supported the arguments for a restricted random distribution pattern proposed by him. Hilditch (53) in reply to Kartha's paper cast doubt on the validity of some of the experimental data and disagreed with Kartha's analyses of existing data.

In their studies of the trisaturated glycerides of milk fat, Boatman *et al.* (10) found that the amount of trisaturated glyceride was found to agree well with the amount calculated by random distribution and there was no preferential selection or exclusion of any of the major saturated fatty acids from the trisaturated glycerides. However, using pancreatic lipase hydrolysis and gas chromatographic analysis of two butter fat samples, Ast and Vander Wal (1) showed that the individual acyl groups were not dispersed at random among the glycerol carbons. When considered only as saturated or unsaturated, and not as individuals, they appeared to be distributed intermolecularly at random.

Successful fractionation of natural triglyceride mixtures by gas chromatography (66) has permitted a new approach to

the determination of butter fat triglyceride structure. Application of gas chromatographic techniques to the analysis of butter fat gave evidence in favour of a non-random fatty acid distribution (67, 68). Further, by thin-layer chromatographic techniques and via lipase hydrolysis, Blank and Privett (7) indicated that the fatty acids were not distributed randomly among the triglycerides of this fat. Recently, Smith et al. (104) fractionated cow milk fat triglycerides by low-temperature crystallization from pentane, followed by countercurrent distribution. Selected fractions were analyzed for fatty acid composition by gas chromatography, and for the positions of individual fatty acids within the triglycerides by the pancreatic lipase technique. The results confirmed the non-random distribution of most of the major fatty acids in milk fat.

II. PARTIAL GLYCERIDES

Monoglycerides and diglycerides contain only one and two fatty acids, respectively, and consequently have two and one free hydroxyl groups, respectively. They do not occur in significant quantities in natural fats unless the fats have been partially hydrolyzed. However, there is some evidence that small quantities are present in essentially all of the natural fats (80). It was suggested that the monoglycerides content of milk and milk products results from lipolysis (57).

Patton and McCarthy (88) assumed that mammary tissue should contain small quantities of intermediate milk lipids, since its net

synthesis of lipids is substantial. In comparison to the composition of milk lipids, the mammary tissue lipids showed elevated levels of all lipid classes except triglycerides. It was found that the substantial levels of diglycerides in the mammary tissue lipids have the 1, 2-configuration. The evidence suggests that the diglycerides were intermediates in triglyceride formation rather than artifacts of lipolysis.

The presence of small amounts of monoglycerides and substantial amounts of diglycerides were demonstrated in milk fat (58, 82). Boudreau and deMan (13) indicated that fresh milk fat contained from 4.4 - 6.6% of diglycerides. They concluded from their study of the composition of milk fat diglycerides and partial glycerides obtained by pancreatic-lipase hydrolysis that diglycerides were formed in the synthesizing cells of the mammary gland. The presence of mono- and diglycerides in milk fat seems to be well established at this time.

III. CHOLESTEROL

Cholesterol is by far the oldest recognized and also the most important member of the sterol group. This alcohol is widely distributed in the animal kingdom but it is completely absent from the plant world. Cholesterol occurs in nature both free and in the form of esters of fatty acids. Like other lipids, cholesterol is soluble in organic solvents as well as in fats and oils. It readily dissolves in organic solvents, this fact enables it to be easily separated from other lipids.

Cholesterol is synthesized in the lactating mammary gland, as demonstrated by the rapid incorporation into these milk lipids of injected carboxyl-C^{14} acetate, both in the intact lactating goat (90) and in the perfused isolated udder of the cow (20). It occurs in the mammary gland at a level of about 0.7% of the dry weight (9). Although Nataf et al. (83) found no evidence of cholesterol ester in milk, Neiman and Groot (84) reported values for them. They concluded from their study of the free and ester cholesterol content of butter that there was esterified cholesterol present in butter. They also found that in one milk sample about 9% of the total cholesterol present was esterified. The situation was complicated by a report of Kritchevsky and Tepper (65). They found no ester cholesterol in milk but large proportions of ester cholesterol in varies dairy products. The presence of small amounts of ester cholesterol in milk and dairy products was clarified by a report of deMan (22). He also indicated that 5 - 10% of the cholesterol of milk fat was present in the ester form. Further, Patton and McCarthy (78, 87) have demonstrated that ester cholesterol is synthesized, at least in part, within the mammary gland.

IV. METHODS FOR THE FRACTIONATION OF FATS

A. Fractional Crystallization

Fractional crystallization from solvents was one of the earliest techniques applied to the separation of triglyceride mixtures. The separation depends upon the relative degree of unsaturation

of the triglycerides. As mentioned previously GS_3, GS_2U, GSU_2 and GU_3 are four solubility types which may occur in any fat. For many years Hilditch and co-workers (54) have used solvent crystallization for the partial separation of these mixtures in connection with their work on glyceride structure of fats.

Acetone has been the most widely used solvent, and crystallizations have commonly been performed at glyceride concentrations of about 10% w/v. The crystallization is conducted at a succession of different temperatures. Ordinarily, the progression is from room temperature toward lower temperature. However, in dealing with materials which come to equilibrium slowly, Hilditch and co-workers (54) claim that it is preferable to work from the lowest temperature toward higher temperatures, to avoid lengthy periods of equilibration.

When fractionation is complete, the percentages of the component acids in each fraction are determined and the glyceride compositions calculated on the assumption that no fraction contains more than two contiguous members of the four main glyceride classes. However, there is evidence that this assumption may not be justified where several short-chain and unsaturated acids are present. It suffers from the disadvantages of being time-consuming and requiring comparatively large samples of the fats and large volumes of solvents (18).

B. Countercurrent Distribution

In countercurrent distribution the substance to be fractionated is subjected to repeated partitioning between two immiscible liquids. Separation has been largely on the basis of the degree of unsaturation of the triglycerides present. Although an automatic 200-tube countercurrent-distribution instrument afforded an opportunity to apply the high resolving power of this instrument to the separation of the complex glyceride mixtures, complete separation into individual triglycerides has not been obtained (27, 94, 95, 96).

Countercurrent distribution possesses much higher resolution than fractional crystallization. However, the fully automatic apparatus is expensive, and solvent consumption is high. This method is not widely used.

C. Thermal Gradient Fractionation

In 1956 Baker and Williams (4) described an ingenious apparatus which automatically separates glycerides by repeated fractional crystallization.

The technique consists of the gradient elution of the mixture from a column which is maintained at a higher temperature at the top and a lower temperature at the bottom. As the sample components dissolve at the top in the increasingly more effective solvent they pass down the column. The more soluble components move ahead of the others and are eluted first. Hammond and

co-workers (59, 76) have applied this apparatus and procedure to the determination of glyceride structure. They found it was possible to separate mixtures of both simple and mixed triglycerides.

Thermal gradient analysis has the advantage of producing intact fractions for further analysis. But the application of this principle to the separation of glycerides requires further study (106).

D. Thin-Layer Chromatography

One of the most promising recent developments has been the segregating the glycerides of natural fats into more simple mixtures by the chromatography on thin layers of silica impregnated with silver nitrate.

After de Vries (24, 25) described a new chromatographic adsorbent for the separation of higher fatty acid methyl esters according to their degree of unsaturation or according to the configuration (*cis* or *trans*) of their double bonds, silicic acid impregnated with silver nitrate is being widely used for the analysis of triglycerides. Barrett *et al*. (5) described a procedure involving thin layer chromatography on silica impregnated with silver nitrate, which makes possible the separation of glyceride mixtures into classes according to their degree of unsaturation, and within those classes the resolution of certain isomeric unsaturated glycerides. Further, the analysis of triglycerides by a combination of analytical techniques in conjunction with fractionation by silicic acid impregnated with silver nitrate has been described by Litchfield *et al*. (71), Subbaram and Youngs (105), McCarthy and Kuksis (79) and Blank *et al*. (8).

EXPERIMENTAL METHODS

I. PREPARATION OF MILK FAT

The pure fat was obtained by melting of butter, repeated washing with warm water until the fat became clear and free from non-lipid material, and then drying under vacuum as described by deMan (21).

II. FRACTIONAL CRYSTALLIZATION

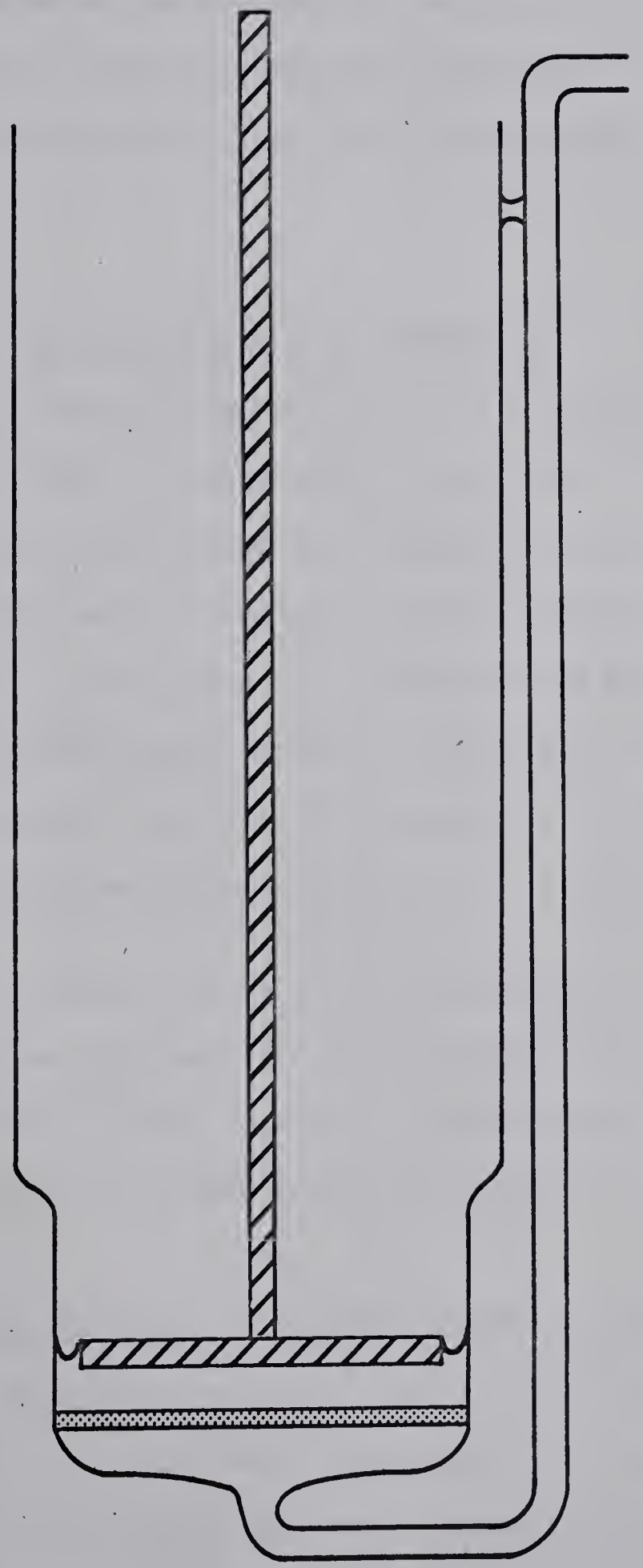
Fractional crystallization from solvent was selected as the separation process for the present study. In the presence of a solvent, equilibrium between the solid and liquid phases is established much more rapidly, large, easily separable crystals are produced even at relatively high cooling rates, and the viscosity of the liquid phase is reduced so that separation of the crystals by filtration is more rapid. If there is a tendency for mixed crystals to form, this tendency is generally reduced in the presence of a solvent. Acetone was chosen as the solvent. Although a number of low melting solvents, including, for example, methyl acetate, methyl alcohol, ethyl alcohol, and petroleum ether, have been used for glyceride and fatty acid separations, acetone is the solvent most commonly employed.

100 g of milk fat was dissolved in 1000 ml of acetone. The fractionation process was performed at temperatures from +15°C to -45°C with the remaining filtrate taken as the final fraction.

The temperature was successively reduced by 10°C decrements and the precipitate that was formed during each temperature interval was separated by filtration. The first two fractions were obtained at +15°C and +5°C respectively. The solution was cooled to +15°C and kept at this temperature in an incubator overnight. The insoluble fraction was removed by filtration. The filtrate was made up to the volume by adding acetone. It was cooled to +5°C and kept at this temperature overnight. The insoluble fraction was again removed by filtration.

All of the fractions collected below 0°C were obtained by using the "Minus Seventy Thermostat Bath". A special apparatus was constructed to prevent temperature changes during the filtration (Fig. 1). The apparatus was made from an 80 mm coarse fritted glass filter. The plunger, fitted with a teflon gasket, made it possible to keep the acetone solution in the apparatus until ready for filtration. The cooling medium in the bath was methanol which was in its turn cooled with a dry ice and methanol mixture. The bath was at the desired temperature before the apparatus, which contained the solution to be crystallized, was put in it. Generally, a period of about 1 hour was required for the solution to be crystallized to attain the desired temperature. After the solution attained the desired temperature, it was kept at that temperature for 3 hours. After 3 hours, the plunger was pulled up and the filtration was achieved by applying vacuum to the side tube. The crystals were washed with small amounts of acetone which had the same temperature as the

Fig. 1. Schematic representation of the filtering apparatus used for the crystallization and filtration of milkfat fractions at controlled temperatures.



solution to be filtered. After filtration, the apparatus was removed from the bath. The crystals were melted and collected. After each filtration the solution was adjusted so that the total volume of solution remained at one liter, leaving temperature as the only variable. The separated fractions were freed of solvent under vacuum.

III. DETERMINATION OF THE FATTY ACID COMPOSITION

The fatty acid composition of the original milk fat and the milk fat fractions were determined as described by deMan (23). The methylation was accomplished in a sealed freeze-drying bulb to prevent loss of the volatile and water soluble methyl esters of short-chain fatty acids. Two volumes of 0.025N potassium methylate in anhydrous methanol were added to one part by weight of fat. The sealed bulb was placed in an oven at 60°C for 1 hour. The change of two-phase to a one-phase system was the sign of complete methylation.

After opening the bulb the esterification mixture was used without further treatment for injection into the column of an F & M Model 720 dual-column temperature-programmed gas chromatograph. Results are expressed as weight percent of methyl ester.

IV. SEPARATION OF GLYCERIDES BY CHROMATOGRAPHY ON FLORISIL

Chromatography on silicic acid is widely used for the separation of tri-, di-, and mono- glycerides, but it has certain disadvantages, and the separations are not always reliable even if

particular care is taken (55). Separations can be achieved in much shorter times with smaller volumes of eluting solvents by using Florisil (Magnesium silicate) which has the further advantage that free fatty acids are eluted after the various glyceride fractions and this eliminates the possibility of their overlapping with tri- or diglycerides (28, 55).

It was found that activated Florisil had a greater adsorption strength and would not give satisfactory separations. It was therefore deactivated by mixing with a measured volume of water (15).

Florisil was used for the separation of glycerides as described by Carroll (15). Florisil was deactivated by mixing with 7% of water (v/w), and 18 g was packed as slurry in hexane in a column (40 x 1 cm). The load was 50 - 150 mg. The eluting solvents were 100 ml of 15% ethyl ether in hexane, followed by 90 ml of 50% ethyl ether in hexane, then by 100 ml of 3% methanol in ethyl ether. These solvents eluted tri-, di-, and monoglycerides in that order.

V. CHOLESTEROL DETERMINATION

Since the use of digitonin for cholesterol assay by Windaus (107), this reagent has been widely used, though it is well known that the digitonin precipitation is not a specific reaction for cholesterol (60, 97). Kabara *et al.* (61) have reported that tomatine is more specific than digitonin in cholesterol precipitation. For the present study, tomatine was used as precipitating agent for the colorimetric determination of cholesterol as described by Kabara

et al. (61). The absorbance of the tomatinide solution was measured with a Bausch and Lomb Spectronic 20 instrument at a wavelength of 625 mμ. It was found that the reading should be made 30 minutes after the Liebermann-Burchard color reagent was added, because the color is stable at this point (22, 45).

Free cholesterol was determined as described by Kabara et al. (61). A weighed sample of milk fat was dissolved in 10 ml acetone-alcohol-ether (4:4:1) and 1% tomatine reagent added. The amount of tomatine reagent added was six times the amount of free sterol present in the sample. After precipitation was complete the tomatinide mixture was centrifuged. The supernatant fluid was decanted and the precipitate washed with acetone-alcohol-ether solution. The washed tomatinide was recentrifuged and rewashed with acetone-ether (1:2).

Hydrolysis of the esterified cholesterol was accomplished by adding 1 or 2 drops of 50% potassium hydroxide to the sample, which was dissolved in acetone-alcohol-ether, and then heating the tubes in sand in a previously warmed jar which was covered tightly and placed in a 60°C incubator for 60 minutes. After saponification the sample was removed from the sand bath and allowed to cool to room temperature before neutralizing to phenophthalein with 10% acetic acid. The resulting solution was then treated as described under free sterol precipitation except that the precipitate was washed only once with ether (45).

The tubes containing the precipitated and washed cholesterol tomatinide were placed in a 110° to 115°C oven. After 30 minutes the tubes were removed and, while still hot 1 ml of glacial acetic acid added from a pipette. After the precipitate was dissolved, the solution was cooled to room temperature. The time was noted and 2 ml of the cold Liebermann-Burchard color reagent added for colorimetric determination (45). The unknown cholesterol value was obtained by using the cholesterol standard curve.

VI. TRISATURATED GLYCERIDE DETERMINATION

Trisaturated glycerides were determined by a method based on the addition of mercuric acetate to the double bonds of the unsaturated triglycerides. The reacted unsaturated triglyceride fraction was absorbed on a Florisil column, and the trisaturated glyceride fraction eluted unchanged and quantitatively. This work was done by Kerkhoven (64).

RESULTS

Because of the mixed nature of glycerides of milk fat, it is extremely difficult to isolate single glycerides or to effect a partial separation of the many individual glycerides in milk fat by fractional crystallization. Table 2 lists the approximate yield of the various fractions of July and December milk fats obtained by fractional crystallization from acetone. The yield was highest for the -15°C fraction. It is interesting to note that the yield of each fraction increased with decreasing melting point to a maximum in the -15°C fraction, then decreased with decreasing melting point to a minimum in the -45°C fraction. The fatty acid compositions of July and December milk fats and their glyceride fractions are listed in Table 3 and 4 respectively. No butyric acid was found in the +15°C fractions. As the melting point decreased, there was an increase in the weight percentage of short-chain fatty acids (particularly butyric acid) and unsaturated fatty acids and a decrease in palmitic and stearic acids. Myristic acid was relatively constant in all of the fractions. It was found that the fatty acid composition of the -15°C fraction was similar to that of the original milk fat. The decrease in palmitic acid content with decreasing melting point of the fractions is not in agreement with the findings of Dimick and Patton (26). They found that the concentration of palmitic acid remained relatively constant throughout all the fractions prepared from milk fat by silicic acid column chromatography.

The trisaturated glyceride (GS_3) contents of July fat and its fractions were determined. Results of these determinations and of the fatty acid compositions of the trisaturated glycerides of the fat and its fractions are listed in Table 5. The differences in the concentration of the various fatty acids between the trisaturated glyceride fractions are similar to that observed in the original July fat and its fractions. As the melting point decreased, there was an increase in the weight percentage of short-chain fatty acids and a decrease in palmitic and stearic acids.

When the component fatty acids of the trisaturated glycerides and of the whole fat are determined, the fatty acid composition of the unsaturated triglycerides can be obtained by difference. This may give a certain degree of insight into the structure of the unsaturated triglycerides of the fat. The +15°C, +5°C, and the residual fractions were chosen for detailed study because the +15°C and the +5°C fractions contained lower proportions of triglycerides with short-chain fatty acids and unsaturated fatty acids than did the original milk fat and the residual fraction contained higher proportions of triglycerides with short-chain and unsaturated fatty acids than did the original milk fat. Furthermore, the +5°C fraction had a higher GS_3 content than the +15°C fraction. It was of interest to know the fatty acid distribution in the +5°C fraction. Tables 6, 7, and 8 give the detailed results of +15°C, +5°C, and residual fractions prepared from July fat. The amount of short-chain fatty acids in the trisaturated glycerides of +5°C fraction

was higher than the amount in the original fraction (Table 7). However, the short-chain fatty acids were found almost entirely in the tri-saturated glycerides in the higher melting fractions and very high amounts in the unsaturated triglycerides of the lower melting fractions.

Table 9 gives the weight percentages of GS_3 of July fat and its fractions, together with the values calculated from the fatty acid composition as given in Table 3. On the whole, the experimental values for the trisaturated glycerides are higher than the calculated values. When the ratios of the concentrations of the major fatty acids in the trisaturated glycerides of July milk fat and its fractions to that in the original fat and its fractions were calculated, a constant ratio was found for the major fatty acids in the original fat and -15°C fraction and a high ratio was found for the short-chain fatty acids in the lower melting fractions. These results are given in Table 10.

As milk fat contains partial glycerides and both free and ester cholesterol it was of interest to determine the distribution of these constituents in the various fractions. Table 11 lists the distribution of partial glycerides in each fraction. As a result of the different solubilities of partial glycerides and triglycerides, the partial glyceride content increased with decreasing melting point of the fractions. The results of free and ester cholesterol analyses are listed in Table 12. It was found that only trace amounts of free cholesterol were present in the higher melting fractions. The amount increased with decreasing melting point and accumulated largely

in the residual fraction. The ester cholesterol had a different distribution pattern. There was no ester cholesterol in the higher melting fractions. The amount found in each fraction did not increase according to the melting point of the fractions.

Table 2. Yield (in weight %) of fractions obtained by fractional crystallization of milk fat from acetone.

Fraction	July fat	December fat
+15°C	8.7	8.6
+5°C	9.7	11.6
-5°C	14.3	15.2
-15°C	27.1	28.7
-25°C	13.4	10.3
-35°C	9.0	9.2
-45°C	4.8	5.0
Residual	13.0	11.4

Table 3. Component acids (in weight %) in the July milk fat and the fractions obtained from it by fractional crystallization from acetone.

	Original fat	+15°C	+5°C	-5°C	-15°C	-25°C	-35°C	-45°C	Residual
% (wt) of whole fat		8.7	9.7	14.3	27.1	13.4	9.0	4.8	13.0
Component acids									
4:0	2.53	-	1.29	3.18	3.93	4.4	4.25	4.92	7.32
6:0	1.55	trace	0.86	1.78	2.12	2.46	2.55	2.84	3.54
8:0	1.03	trace	0.52	0.93	1.24	1.61	1.7	1.84	2.14
10:0	2.42	0.93	1.52	1.87	2.54	3.52	3.45	3.35	3.45
10:1	0.24	-	-	0.16	0.24	0.28	0.36	0.43	0.47
12:0	2.92	2.47	2.60	2.07	3.3	4.09	3.56	3.61	3.10
14:0	10.13	11.19	9.26	9.54	12.63	10.0	7.92	8.36	7.56
14:1 15:0 + 16:0^{i}	2.44	2.11	2.59	2.34	2.5	3.09	2.87	3.12	2.78
16:0	23.36	33.85	32.90	29.74	23.92	16.90	17.48	14.76	9.81
16:1 17:0 + 17:0^{i}	2.88	2.88	2.66	2.59	2.9	2.84	3.86	4.13	3.70
18:0	14.28	28.58	26.93	20.12	11.83	8.69	6.73	6.00	1.93
18:1	29.47	14.43	16.17	20.97	26.79	35.04	37.06	38.48	44.95
18:2	3.91	3.28	2.05	3.49	4.51	4.36	5.15	5.19	6.33
18:3	2.84	0.28	0.65	1.22	1.45	2.61	2.96	2.82	2.62
a	0	0	0	0	0	0	0	0	0
b	0	0	0	trace	0.1	0.11	0.1	0.15	0.30
Total S	63.54	82.01	81.13	74.16	67.01	57.71	54.47	53.08	45.63
Total U	36.46	17.99	18.87	25.84	32.99	42.29	45.53	46.92	54.37

a. Fatty acid having retention volumes between 4:0 and 6:0
b. Fatty acid having retention volumes between 8:0 and 10:0

Table 4. Component acids (in weight %) in the December milk fat and the fractions obtained from it by fractional crystallization from acetone.

	Original fat	+15°C	+5°C	-5°C	-15°C	-25°C	-35°C	-45°C	Residual
% (wt) of whole fat		8.6	11.6	15.2	28.7	10.3	9.2	5.0	11.4
Component acids									
4:0	2.36	-	1.28	3.76	3.17	3.31	3.79	4.93	6.99
6:0	1.50	trace	0.79	1.99	1.78	1.98	2.48	2.73	3.16
8:0	0.99	trace	0.55	1.07	0.97	1.17	1.49	1.61	2.16
10:0	2.26	0.56	1.40	1.89	2.15	2.35	3.12	2.80	2.97
10:1	0.32	-	trace	0.21	0.26	0.52	0.46	0.36	0.33
12:0	2.81	1.64	2.01	1.79	2.99	3.79	3.3	3.32	2.99
14:0	9.73	10.69	8.75	9.81	11.8	10.76	7.73	8.16	8.44
14:1 15:0 + 16:0^{i}	2.08	2.2	2.18	2.96	3.26	3.63	3.45	4.12	3.71
16:0	25.72	37.88	36.46	34.96	28.78	22.17	18.01	16.11	11.97
16:1 17:0 + 17:0^{i}	3.96	1.55	3.06	3.79	3.57	3.21	3.51	5.02	5.37
18:0	11.96	29.89	22.84	14.68	10.88	6.68	7.56	6.20	1.66
18:1	28.38	9.66	17.57	19.58	26.66	35.89	39.47	39.36	43.48
18:2	5.71	3.84	1.84	3.08	2.76	2.82	4.22	4.57	5.55
18:3	2.22	2.09	1.27	0.43	1.07	1.22	1.41	0.71	1.22

Table 5. Component acids (in weight %) in the trisaturated glycerides (GS_3) of July milk fat and of fractions obtained from it by fractional crystallization from acetone*.

	Original fat	+15°C	+5°C	-5°C	-15°C	-25°C	-35°C	-45°C	Residual
% (wt) of whole fat	38.4	67.2	68.6	52.3	43.7	32.2	30.6	27.5	27.2
Component acids									
4:0	4.3	-	2.3	4.9	5.1	5.7	5.8	6.5	7.1
6:0	2.3	trace	1.4	2.9	2.9	3.5	4.2	4.6	5.3
8:0	1.5	trace	0.8	1.4	1.5	2.1	2.9	3.5	4.5
10:0	3.6	0.6	2.4	3.0	3.6	5.5	7.6	7.8	9.1
12:0	4.6	2.4	3.5	3.0	4.7	7.9	9.4	9.0	8.0
14:0	15.8	12.2	11.9	10.3	18.0	20.8	16.9	17.0	16.0
15:0 + $16:0^i$	5.2	1.3	3.6	2.8	3.9	5.1	4.7	4.9	3.9
16:0	36.1	41.0	39.0	41.6	39.3	31.5	29.8	28.7	19.4
17:0 + $17:0^i$	2.8	2.7	2.2	2.9	1.9	1.4	2.7	2.8	2.2
18:0	22.4	39.9	32.9	26.5	17.7	13.4	12.0	12.6	6.5
a	0.6	0	0	0	0.1	0.1	0.2	0.4	0.5
b	0.2	0	0	0.7	1.1	2.2	3.0	1.6	16.8
c	0	0	0	0	trace	0.2	0.4	0.2	0.3
d	0.4	0	0	0	0.2	0.6	0.5	0.4	0.4

a. Fatty acid having retention volumes between 4:0 and 6:0
b. Fatty acid having retention volumes between 8:0 and 10:0
c. Fatty acid having retention volumes slightly smaller than 10:1
d. Fatty acid having retention volumes between 10:0 and 12:0

*Data of Kerkhoven (64).

Table 6. Fatty acid distribution in the +15°C fraction and in the trisaturated glycerides and the unsaturated triglycerides obtained from it.

Acid	Weight % of acids in			Weight % of +15° fraction due to glyceride increments	
	+15°C fraction	Trisaturated glycerides (detd)	Unsaturated triglycerides (calcd)*	Trisaturated glycerides (67.2%)	Unsaturated triglycerides (32.8%)
4:0	-	-	-	-	-
6:0	trace	trace	-	trace	-
8:0	trace	trace	-	trace	-
10:0	0.93	0.6	1.59	0.4	0.53
10:1	-	-	-	-	-
12:0	2.47	2.4	2.62	1.61	0.86
14:0	11.19	12.2	9.09	8.2	2.99
14:1 15:0 + 16:0^{i}	2.11	1.3	3.78	0.87	1.24
16:0	33.85	41.0	19.21	27.55	6.30
16:1 17:0 + 17:0^{i}	2.88	2.7	3.26	1.81	1.07
18:0	28.58	39.9	5.37	26.81	1.77
18:1	14.43	-	43.99	-	14.43
18:2	3.28	-	10.0	-	3.28
18:3	0.28	-	0.85	-	0.28

*From the (a) determined composition of the +15° fraction and its trisaturated glycerides and (b) weight percentages of trisaturated glycerides in the +15° fraction.

Table 7. Fatty acid distribution in the +5°C fraction and in the trisaturated glycerides and the unsaturated triglycerides obtained from it.

Acid	Weight % of acids in			Weight % of +5°C fraction due to glyceride increments	
	+5°C fraction	Trisaturated glycerides (detd)	Unsaturated triglycerides (calcd)*	Trisaturated glycerides (68.6%)	Unsaturated triglycerides (31.4%)
4:0	1.29	2.3	-0.9	1.58	-0.28
6:0	0.86	1.4	-0.3	0.96	-0.1
8:0	0.52	0.8	-0.1	0.55	-0.03
10:0	1.52	2.4	-0.4	1.65	-0.13
10:1	-	-	-	-	-
12:0	2.60	3.5	0.63	2.40	0.2
14:0	9.26	11.9	3.50	8.16	1.1
14:1 15:0 + 16:0^{i}	2.59	3.6	0.38	2.47	0.12
16:0	32.90	39.0	19.59	26.75	6.15
16:1 17:0 + 17:0^{i}	2.66	2.2	1.46	1.51	0.46
18:0	26.93	32.9	13.89	22.57	4.36
18:1	16.17	-	51.5	-	16.17
18:2	2.05	-	6.53	-	2.05
18:3	0.65	-	2.07	-	0.65

*From the (a) determined composition of the +5°C fraction and its trisaturated glycerides and (b) weight percentages of trisaturated glycerides in the +5°C fraction.

Table 8. Fatty acid distribution in the residual fraction and in the trisaturated glycerides and the unsaturated triglycerides obtained from it.

Acid	Weight % of acids in			Weight % of residue due to glyceride increments.	
	Residual fraction	Trisaturated glycerides (detd)	Unsaturated triglycerides (calcd)*	Trisaturated glycerides (27.2%)	Unsaturated triglycerides (72.8%)
4:0	7.32	7.1	7.4	1.93	5.39
6:0	3.54	5.3	2.88	1.44	2.10
8:0	2.14	4.5	1.26	1.22	0.92
10:0	3.45	9.1	1.33	2.48	0.97
10:1	0.47	-	0.65	-	0.47
12:0	3.10	8.0	1.26	2.18	0.92
14:0	7.56	16.0	4.41	4.35	3.21
14:1 15:0 + 16:0^{i}	2.78	3.9	2.36	1.06	1.72
16:0	9.81	19.4	6.22	5.28	4.53
16:1 17:0 + 17:0^{i}	3.70	2.2	4.26	0.6	3.10
18:0	1.93	6.5	0.22	1.77	0.16
18:1	44.95	-	61.74	-	44.95
18:2	6.33	-	8.7	-	6.33
18:3	2.62	-	3.6	-	2.62

*From the (a) determined composition of the residual fraction and its trisaturated glycerides and (b) weight percentages of trisaturated glycerides in the residual fraction.

Table 9. Content of trisaturated glycerides in the July milk fat and the fractions obtained from it by fractional crystallization from acetone.

Sample	Experimental % (wt) trisaturated glycerides	% (wt) calculated on basis of random distribution
July fat	38.4	25.65
+15°C fraction	67.2	55.1
+5°C fraction	68.6	53.4
-5°C fraction	52.3	40.8
-15°C fraction	43.7	30.1
-25°C fraction	32.2	19.2
-35°C fraction	30.6	16.2
-45°C fraction	27.5	15.0
Residue	27.2	9.5

Table 10. Ratios of weight % of the major fatty acids in the trisaturated glycerides of July milk fat and its fractions to that of the fatty acids in the original milk fat and its fractions.

Fatty acid	Original fat	Fraction obtained at °C							
		+15	+5	-5	-15	-25	-35	-45	Residue
4:0	1.53	-	0.82	1.24	1.76	2.39	2.40	2.75	3.79
6:0	1.76		0.9	1.17	1.67	2.18	1.99	2.28	2.47
8:0	1.78		0.95	1.27	1.88	2.37	1.91	1.92	1.77
10:0	1.75	2.30	0.92	1.19	1.62	1.99	1.49	1.56	1.42
12:0	1.65	1.53	1.08	1.32	1.61	1.61	1.24	1.46	1.44
14:0	1.67	1.36	1.13	1.77	1.60	1.50	1.54	1.79	1.75
16:0	1.69	1.23	1.23	1.37	1.40	1.67	1.92	1.88	1.86
18:0	1.66	1.07	1.19	1.45	1.54	2.02	1.83	1.73	1.10

Table 11. Mono-, di-, and tri- glyceride contents (in weight %) of July and December milk fats and the fractions obtained from them by fractional crystallization from acetone.

Sample	Monoglycerides		Diglycerides		Triglycerides	
	July	December	July	December	July	December
Milk fat	0.84	0.74	6.97	8.08	92.19	91.17
+15°C fraction	0.47	0.64	2.29	2.51	97.23	96.85
+5°C fraction	0.52	0.65	3.64	4.56	95.84	94.79
-5°C fraction	1.05	1.69	5.71	6.41	93.24	91.9
-15°C fraction	1.34	1.82	9.54	7.62	89.12	90.56
-25°C fraction	1.56	1.03	7.79	11.07	90.65	87.9
-35°C fraction	1.66	1.53	9.35	13.33	88.99	85.14
-45°C fraction	2.79	2.75	16.56	20.42	80.65	76.83
Residue	2.6	2.12	14.81	19.78	82.59	78.10

Table 12. Free, ester, and total cholesterol contents of July and December milk fats and the fractions obtained from them by fractional crystallization from acetone.

	Cholesterol concentrations mg/100 g fat					
	Free		Ester*		Total	
Sample	July	December	July	December	July	December
Milk fat	209	210	27	47	236	257
+15°C fraction	trace	trace	-	-	trace	trace
+5°C fraction	trace	trace	-	-	trace	trace
-5°C fraction	20	11	40	57	60	68
-15°C fraction	33	40	60	60	93	100
-25°C fraction	125	129	31	50	156	179
-35°C fraction	136	117	35	61	171	178
-45°C fraction	130	180	28	59	158	239
Residue	972	1164	48	99	1020	1263

* Ester cholesterol was calculated by difference.

DISCUSSION

I. FRACTIONAL CRYSTALLIZATION FROM SOLVENT

Fractional crystallization from solvent has advantages in that it is easy to perform and the molecules are not disrupted by the process. It suffers from the disadvantages of being time-consuming and of the lack of a sharp distinction between the solubility characteristics of the various components. Furthermore, difficulty is encountered in this procedure in the efficient removal of entrained filtrate in the crystal fraction.

There are four types of mixed triglycerides, GS_3, GS_2U, GSU_2, and GU_3 as mentioned previously. In the majority of fats (in which saturated acids of smaller molecular size than palmitic or myristic are not present) the crystallization procedure causes all of the trisaturated glycerides to be concentrated in the most sparingly soluble fractions (e.g. +15, +5). Milk fat is exceptional in this respect because mixed trisaturated glycerides containing two short-chain acyl groups in addition to one of the more usual long-chain groups are still very soluble in acetone and in consequence trisaturated glycerides appear throughout all of the separated fractions of the milk fat. Milk fat contains about 10 mole percent of butyric acid which causes the triglycerides to crystallize approximately at the same temperature as when unsaturated acid were present at the same position of the triglycerides.

Therefore, unsaturated triglycerides also appear throughout all the separated fractions. The assumption that no fraction contains more than two contiguous members of the four main glyceride classes is not justified in the case of milk fat.

II. THE COMPONENT GLYCERIDES

From a consideration of the fatty acid composition of each fraction obtained by fractional crystallization and the corresponding trisaturated glycerides, the general pattern of milk fat glycerides can be pictured by examination of the relative occurrence of palmitic, stearic, oleic and short-chain fatty acids.

From detailed study of the +5°C and residual fraction, given in Tables 7 and 8, it can be concluded that the short-chain fatty acids in the high melting glycerides are combined with palmitic or stearic acid. In contrast, in the low melting glycerides, short-chain fatty acids are combined with unsaturated fatty acids. This is in accord with the findings of Kuksis et al. (67). These investigators concluded that the short-chain fatty acids as butyric and caproic were found exclusively in combination with medium and long chain fatty acids in the glyceride molecules.

There are high levels of palmitic and stearic acid in the GU of the +5°C fraction and relatively high amounts of myristic and palmitic acid in the GU of the residual fraction. There are relatively high amounts of myristic, palmitic, and stearic acids in the GU of the +15°C fraction (Table 6). It can be concluded

that the higher molecular weight saturated acids tend to be associated in the triglycerides with unsaturated fatty acid, mainly oleic acid. This is in agreement with the conclusions of Smith and co-workers (36, 104). They found that the higher molecular weight saturated acids tend to be associated in the triglycerides with monoethenoid acids. The relatively high amounts of myristic and very high amounts of palmitic and stearic acid in the GS_3 of the +15°C fraction indicate that myristic acid is combined with both palmitic and stearic acids to form the high melting glyceride.

It is to be expected that the GS_3 content of each fraction will decrease as the melting point decreases. The +5°C fraction was, however, an exception in this respect as it had a little higher GS_3 content than the +15°C fraction. The detailed results, given in Table 7, show that the amount of short-chain fatty acids in the trisaturated glycerides was higher than the amount in the original fraction, this may be explained as the result of experimental error.

The weight percentage of trisaturated glycerides in the July milk fat was found to be 38.4% of the total glycerides. This determined value is not close to the theoretical value for the trisaturated glycerides, based on a completely random distribution of the saturated acids in the formation of the glycerides. The theoretical value for the weight percent of trisaturated glyceride, assuming random distribution of 63.54% saturated acids, is $\frac{(63.54)^3}{(100)^3} \times 100 = 25.65\%$. Table 9 also indicates that the experimental GS_3 values for the fractions are not close to the theoretical

values. It must be concluded that the distribution pattern of the fatty acids among the glycerides of milk fat and its fractions do not conform to a random distribution pattern.

The ratios of the concentrations of the major fatty acids in the GS_3 of milk fat to that in the original fat were constant. This is in agreement with the conclusion of Boatman et al. (10) that there is no preferential selection or exclusion of any of the major saturated fatty acids from the trisaturated glycerides. The ratios for the separated fractions were not constant except for the -15°C fraction. It seems that the distribution pattern of the fatty acids among the glycerides was changed by fractional crystallization from acetone. The ratios of the -15°C fraction and the original milk fat were similar. This may be explained by their similar fatty acid compositions.

On the basis of the results presented in Table 3 and 4 the glycerides may be divided into three groups. The first group is composed of the first two fractions. It comprises about 18.4% and 20.2% of the whole fat for July and December milk fat respectively. These fractions are characterized by being low in short-chain fatty acids and high in saturated fatty acids. The second group is composed of the next three fractions. They make up 54.8% and 54.2% of the whole fat for July and December milk fat respectively. They contain more short-chain and unsaturated fatty acids. These fractions are similar in composition to the original fat. The third group is composed of the fractions obtained at the lowest temperatures and

these glycerides are high in both short-chain and unsaturated fatty acids. This is in accord with the findings of Cantabrana and deMan (14). By differential thermal analysis they found that three major peaks were present in the melting curves of milk fat and these indicated the presence of three major glyceride groups.

III. PARTIAL GLYCERIDES AND CHOLESTEROL

Because of the mixed nature of glycerides of milk fat it is to be expected that different partial glycerides will have different solubilities and different melting points and as a consequence partial glycerides appear in all of the separated fractions. Partial glycerides are more soluble than triglycerides, therefore, the partial glyceride content of each fraction increased with decreasing melting point.

Although it was found by Clément *et al.* (16) that the separation of sterols from glycerides by differential solubilities was not satisfactory, Oliver *et al.* (85) removed sterols from peanut oil by crystallization from acetone at a low temperature. Cholesterol is more soluble than the other constituents. Only trace amounts of free cholesterol were found in the higher melting fractions. The free cholesterol content in each fraction increased with decreasing melting point and finally accumulated largely in the residual fraction. Ester cholesterol was calculated by difference. Since only trace amounts of total and free cholesterol were found in the higher melting fractions, there was no ester cholesterol present in the higher

melting fractions. As shown in Table 12, ester cholesterol had a different distribution pattern among the separated fractions. The ester cholesterol content in each fraction does not increase according to the melting point of the fractions. It seems that ester cholesterol is less soluble than the free cholesterol, and this is probably dependent on the nature of the esterified fatty acid.

IV. MINOR CONSTITUENTS IN SOME OF THE LOWER MELTING FRACTIONS

The minor constituents which appeared on the chromatograms of some of the lower melting fractions and their trisaturated glycerides are not normally visible on the chromatograms of the whole fat. As odd carbon number short-chain fatty acids have been identified in milk fat (47), these minor constituents are presumably odd carbon number short-chain fatty acids which were enriched in some of the fractions.

BIBLIOGRAPHY

1. AST, H.J., and VANDER WAL, R.J.
The structural components of milk triglycerides.
J. Am. Oil Chem. Soc., 38: 67. (1961).

2. AYLWARD, F.X., BLACKWOOD, J.H., and SMITH, J.A.B.
Lipaemia and milk fat secretion in the ruminant.
Biochem. J. 31: 130. (1937).

3. BACKDERF, R.H., and BROWN, J.B.
Further contributions to the nature of the monoethenoic fatty acids of butterfat.
Archs Biochem. Biophys., 76: 15. (1958).

4. BAKER, C.A., and WILLIAMS, R.J.P.
A new chromatographic procedure and its application to high polymers.
J. chem. Soc., p. 2352. (1956).

5. BARRETT, C.B., DALLAS, M.S.J., and PADLEY, F.B.
The quantitative analysis of triglyceride mixtures by thin layer chromatography on silica impregnated with silver nitrate.
J. Am. Oil Chem. Soc., 40: 580. (1963).

6. BERTRAM, S.H.
Biochem. Z. 197: 433. (1928). Cited by Hilditch, T.P., and Williams, P.N. The chemical constitution of natural fats. 4th edition, Chapman & Hall, London (1964).

7. BLANK, M.L., and PRIVETT, O.S.
Structure of milk fat triglycerides.
J. Dairy Sci., 47: 481. (1964).

8. BLANK, M.L., VERDINO, B., and PRIVETT, O.S.
Determination of triglyceride structure via silver nitrate - TLC.
J. Am. Oil Chem. Soc., 42: 87. (1965).

9. BLOOR, W.R.
Biochemistry of the fatty acids and their compounds, the lipids.
Reinhold, New York. (1943).

10. BOATMAN, C., DECOTEAU, A.E., and HAMMOND, E.G.
Trisaturated glycerides of milk fat.
J. Dairy Sci., 44: 644. (1961).

11. BOSWORTH, A.W., and BROWN, J.B.
Isolation and identification of some hitherto unreported fatty acids in butter fat.
J. biol. Chem., 103: 115. (1933).

12. BOSWORTH, A.W., and SISSON, E.W.
Arachidonic acid in butter fat.
J. biol. Chem., 107: 489. (1934).

13. BOUDREAU, A., and deMAN, J.M.
The composition of milkfat diglycerides and partial glycerides obtained by pancreatic-lipase hydrolysis.
Biochim. biophys. Acta, 98: 47. (1965).

14. CANTABRANA, F., and deMAN, J.M.
Differential thermal analysis of the melting and solidification of milk fat.
J. Dairy Sci., 47: 32. (1964).

15. CARROLL, K.K.
Separation of lipid classes by chromatography on florisil.
J. Lipid Res., 2: 135. (1961).

16. CLÉMENT, G., CLÉMENT, J., and LOUEDEC, A.
Sur la séparation des esters de cholestérol á partir d'extraits lipidiques de tissue animaux.
Archs Sci. physiol. 8: 233. (1954). Cited by Cook, R.P. Cholesterol. Academic Press Inc., New York. (1958).

17. CHEVREUL, M.E.
Recherches chimiques sur les corps gras d'origine animale. Levrault, G., Paris 1823. Cited by Ralston, A.W. Fatty acids and their derivatives. John Wiley and Sons, Inc., New York. (1948).

18. COLEMAN, M.H.
The structural investigation of natural fats.
In: Advances in Lipid Research, I, Paoletti, R., and Kritchevsky, D., eds.
Academic Press, New York. (1963).

19. CORNWELL, D.G., BACKDERF, R., WILSON, C.L., and BROWN, J.B.
The trans-octadecenoic acid content of butterfat.
Archs Biochem. Biophys., 46: 364. (1953).

20. COWIE, A.T., DUNCOMBE, W.G., FOLLEY, S.J., FRENCH, T.H., GLASCOCK, R.F., MASSART, L., and PEETERS, G.J.
Synthesis of milk fat from acetic acid by the purfused isolated bovine udder.
Biochem. J., 49: 610. (1951).

21. deMAN, J.M.
Physical properties of milk fat. I. Influence of chemical modification.
J. Dairy Res., 28: 81. (1961).

22. deMAN, J.M.
The free and ester cholesterol content of milk and dairy products.
Z. ErnährWiss, 5: 1. (1964).

23. deMAN, J.M.
Determination of the fatty acid composition of milk fat by dual column temperature programmed gas liquid chromatography.
J. Dairy Sci., 47: 546. (1964).

24. de VRIES, B.
Quantitative separations of lipid materials by column chromatography on silica impregnated with silver nitrate.
Chemy Ind., p. 1049. (1962).

25. de VRIES, B.
Quantitative separations of higher fatty acid methyl esters by adsorption chromatography on silica impregnated with silver nitrate.
J. Am. Oil Chem. Soc., 40: 184. (1963).

26. DIMICK, P.S., and PATTON, S.
Structure and synthesis of milk fat. VII. Distribution of fatty acids in milk fat triglycerides with special reference to butyrate.
J. Dairy Sci., 48: 444. (1965).

27. DUTTON, H.J., and CANNON, J.A.
Glyceride structure of vegetable oils by counter-current distribution. I. Linseed oil.
J. Am. Oil Chem. Soc., 33: 46. (1956).

28. FILLERUP, D.L., and MEAD, J.F.
Chromatographic separation of the plasma lipids.
Proc. Soc. exp. Biol. Med., 83: 574. (1953).

29. GARTON, G.A.
The composition and biosynthesis of milk lipids.
J. Lipid Res., 4: 237. (1963).

30. GLASCOCK, R.F., DUNCOMBE, W.G., and REINIUS, L.R.
Studies on the origin of milk fat. 2. The secretion of dietary long-chain fatty acids in milk fat by ruminants.
Biochem. J., 62: 535. (1956).

31. GRAHAM, W.R., JONES, T.S.G., and KAY, H.D.
The precursors in cows' blood of milk fat and other milk constituents.
Proc. R. Soc. London. B., 120: 330. (1936).

32. GREENBANK, G.R.
The glyceride structure and polymorphism of butterfat.
J. Dairy Sci., 35: 486. (1952).

33. GREENBANK, G.R.
The fractionation and properties of the glyceride fractions of butterfat.
Proc. XIIIth Int. Dairy Congr., 3: 1269. (1953).

34. GRÜN, A., and WIRTH, T.
Decylensäure, eine bisher unbekannte Säure aus der Butter.
Ber. 55: 2197. (1922).
Cited by Hilditch, T.P., and Williams, P.N. The chemical constitution of natural fats. 4th edition, Chapman & Hall, London (1964).

35. GRÜN, A.
Z. angew. Chem. 37: 228. (1924).
Cited by Hilditch, T.P., and Williams, P.N. The chemical constitution of natural fats. 4th edition, Chapman & Hall. London (1964).

36. HAAB, W., SMITH, L.M., and JACK, E.L.
Countercurrent distribution of milk fat triglycerides.
J. Dairy Sci., 42: 454. (1959).

37. HANSEN, R.P., and SHORLAND, F.B.
The branched-chain fatty acids of butterfat. I. The isolation from butterfat of branched-chain fatty acids with special reference to the C_{17} acids.
Biochem. J., 50: 207. (1952).

38. HANSEN, R.P., SHORLAND, F.B., and COOKE, N.J.
The branched-chain fatty acids of butterfat. 5. The isolation of 12-methyltridecanoic acid.
Biochem. J., 58: 358. (1954).

39. HANSEN, R.P., SHORLAND, F.B., and COOKE, N.J.
The occurrence of n-undecanoic acid in butterfat.
Chemy Ind., p. 92. (1955).

40. HANSEN, R.P., SHORLAND, F.B., and COOKE, N.J.
Occurrence in butterfat of n-heptadecanoic acid.
Nature, 179: 98. (1957).

41. HANSEN, R.P., SHORLAND, F.B., and COOKE, N.J.
Isolation from butterfat of 14-methyl pentadecanoic (Isopalmitic) acid.
Chemy Ind., p. 124. (1959).

42. HANSEN, R.P., SHORLAND, F.B., and COOKE, N.J.
Isolation and identification of the high molecular weight saturated fatty acids of butterfat.
J. Dairy Res., 26: 190. (1959).

43. HANSEN, R.P., SHORLAND, F.B., and COOKE, N.J.
The isolation of cis-Δ^9-heptadecenoic acid from butterfat.
Biochem. J., 77: 64. (1960).

44. HANSEN, R.P., and COOKE, N.J.
The isolation and characterization of trans-octadec-16-enoic acid from butterfat.
Biochem. J., 31: 233. (1961).

45. HAWK, P.B., OSER, B.L., and SUMMERSON, W.H.
Practical Physiological Chemistry 13th ed.
McGraw-Hill Book Company, Inc., New York. (1954).

46. HENDERSON, J.L., and JACK, E.L.
The fractionation of milk fat from a solvent at low temperatures.
Oil & Soap, 21: 90. (1944).

47. HERB, S.F., MAGIDMAN, P., LUDDY, F.E., and RIEMENSCHNEIDER, R.W.
Fatty acids of cows' milk. B. Composition by gas-liquid chromatography aided by other methods of fractionation.
J. Am. Oil Chem. Soc., 39: 142. (1962).

48. HILDITCH, T.P., and LEA, C.H.
Investigation of the constitution of glycerides in natural fats. A. Preliminary outline of two new methods.
J. chem, Soc., p. 3106. (1927).

49. HILDITCH, T.P., and THOMPSON, H.M.
The effect of certain ingested fatty oils upon the composition of cow milk fat.
Biochem. J., 30: 677. (1936).

50. HILDITCH, T.P., and PAUL, H.
The occurrence and possible significance of some of the minor component acids of cow milk fat.
Biochem. J., 30: 1905. (1936).

51. HILDITCH, T.P., and LONGENECKER, H.E.
Further determination and characterization of the component acids of butterfat.
J. biol. Chem., 122: 497. (1938).

52. HILDITCH, T.P., and JASPERSON, H.
The influence of dietary fat of varying unsaturation on the component acids of cow milk fats.
Biochem. J., 37: 238. (1943).

53. HILDITCH, T.P.
Letter to the editor.
J. Am. Oil Chem. Soc., 31: 433. (1954).

54. HILDITCH, T.P., and WILLIAMS, P.N.
The chemical constitution of natural fats. 4th edition, Chapman & Hall, London. (1964).

55. HIRSCH, J., and AHRENS, E.H.
The separation of complex lipide mixtures by the use of silicic acid chromatography.
J. biol. Chem., 233: 311. (1958).

56. JACK, E.L., HENDERSON, J.L., and HINSHAW, E.B.
The distribution pattern of fatty acids in glycerides of milk fat.
J. biol. Chem., 162: 119. (1946).

57. JENSEN, R.G., GANDER, G.W., and DUTHIE, A.H.
Total monoglyceride content of some dairy products.
J. Dairy Sci., 42: 1913. (1959).

58. JENSEN, R.G., SAMPUGNA, J., and GANDER, G.W.
Fatty acid composition of the diglycerides from lipolyzed milk fat.
J. Dairy Sci., 44: 1983. (1961).

59. JONES, G.V., and HAMMOND, E.G.
Analysis of the glyceride structure of cocoa butter by thermal gradient crystallization.
J. Am. Oil Chem. Soc., 38: 69. (1961).

60. KABARA, J.J.
A quantitative micromethod for the isolation and liquid scintillation assay of radioactive free and ester cholesterol.
J. Lab. clin. Med., 50: 146. (1957).

61. KABARA, J.J., McLAUGHLIN, J.T., and RIEGEL, C.A.
Quantitative microdetermination of cholesterol using tomatine as precipitating agent.
Analyt. Chem., 33: 305. (1961).

62. KARTHA, A.R.S.
The glyceride structure of natural fats. III. Factors governing the content of fully saturated glycerides.
J. Am. Oil Chem. Soc., 31: 85. (1954).

63. KLEIBER, M., SMITH, A.H., BLANK, A.L., BROWN, M.A., and TOLBERT, B.M.
Acetate as a precursor of milk constituents in the intact dairy cow.
J. biol. Chem., 197: 371. (1952).

64. KERKHOVEN, E.W.
The determination of trisaturated triglycerides.
Thesis. University of Alberta. (1965).

65. KRITCHEVSKY, D., and TEPPER, S.A.
The free and ester sterol content of various foodstuffs.
J. Nutr., 74: 441. (1961).

66. KUKSIS, A., and McCARTHY, M.J.
Gas-liquid chromatographic fractionation of natural triglyceride mixtures by carbon number.
Can. J. Biochem. Physiol., 40: 679. (1962).

67. KUKSIS, A., McCARTHY, M.J., and BEVERIDGE, J.M.R.
Quantitative gas liquid chromatographic analysis of butterfat triglycerides.
J. Am. Oil Chem. Soc., 40: 530. (1963).

68. KUKSIS, A., McCARTHY, M.J., and BEVERIDGE, J.M.R.
Triglyceride composition of native and rearranged butter and coconut oils.
J. Am. Oil Chem. Soc., 41: 201. (1964).

69. KUMAR, S., LAKSHMANAN, S., and SHAW, J.C.
β-Hydroxybutyrate and acetate metabolism of the perfused bovine udder.
J. biol. Chem., 234: 754. (1959).

70. LAURYSSENS, M., VERBEKE, R., and PEETERS, G.
Metabolism of stearate-1-C^{14} in the isolated cow's udder.
J. Lipid Res., 2: 383. (1961).

71. LITCHFIELD, C., FARQUHAR, M., and REISER, R.
Analysis of triglycerides by consecutive chromatographic techniques. I. Cuphea llavia seed fat.
J. Am. Oil Chem. Soc., 41: 588. (1964).

72. LONGENECKER, H.E.
Composition and structural characteristics of glycerides in relation to classification and environment.
Chem. Rev., 29: 201. (1941)

73. LOUGH, A.K., and GARTON, G.A.
Blood Lipids. 2. Plasma lipids of the lactating cow: Fatty acid composition of the sterol esters and triglycerides.
Biochem. J., 67: 345. (1957).

74. LUICK, J.R., and LUCAS, J.M.
Further studies on milk fat synthesis.
Proc. Soc. exp. Biol. Med., 110: 275. (1962).

75. MAGIDMAN, P., HERB, S.F., BARFORD, R.A., and RIEMENSCHNEIDER, R.W.
Fatty acids of cows' milk. A. Techniques employed in supplementing gas-liquid chromatography for identification of fatty acids.
J. Am. Oil Chem. Soc., 39: 137. (1962).

76. MAGNUSSON, J.R., and HAMMOND, E.G.
The separation of glycerides by crystallization in a thermal gradient.
J. Am. Oil Chem. Soc., 36: 339. (1959).

77. McCARTHY, R.D., PATTON, S., and SVANS, L.
Structure and synthesis of milk fat. II. Fatty acid distribution in the triglycerides of milk and other animal fats.
J. Dairy Sci., 43: 1196. (1960).

78. McCARTHY, R.D., and PATTON, S.
Cholesterol esters and the synthesis of milk fat.
Biochim. biophys. Acta, 70: 102. (1963).

79. McCARTHY, M.J., and KUKSIS, A.
Exploitation of the selectivity of various chromatographic techniques for the study of the triglyceride structure of natural fats.
J. Am. Oil Chem. Soc., 41: 527. (1964).

80. MEHLENBACHER, V.C.
The analysis of fats and oils.
The Garrard Press, Champaign, Illinois (1960).

81. MEIGS, E.B., BLATHERWICK, N.R., and CARY, C.A.
Contributions to the physiology of phosphorus and calcium metabolism as related to milk secretion.
J. biol. Chem., 37: 1 (1919).

82. MICKLE, J.B., Von GUNTEN, R.L., and MORRISON, R.D.
Rearrangement of milk fat as a means for adjusting hardness of butterlike products.
J. Dairy Sci., 46: 1357. (1963).

83. NATAF, B., MICKELSEN, O., KEYS, A., and PETERSEN, W.E.
The cholesterol content of cows' milk.
J. Nutr., 36: 495. (1948).

84. NIEMAN, C., and GROOT, E.H.
On cholesterol and cholesterol esters in butter.
Acta Physiol. Pharmac. Neerl, 1: 488. (1950).

85. OLIVER, G.D., SINGLETON, W.S., and BAILEY, A.E.
Molecularly distilled peanut oil antioxidants and pure alpha-tocopherol as stabilizing agents for fats of poor keeping quality.
Oil & Soap, 21: 188. (1944).

86. PATTON, S., McCARTHY, R.D., EVANS, L., and LYNN, T.R.
Structure and synthesis of milk fat. 1. Gas chromatographic analysis.
J. Dairy Sci., 43: 1187. (1960).

87. PATTON, S., and McCARTHY, R.D.
Structure and synthesis of milk fat. IV. Role of the mammary gland with special reference to the cholesterol esters.
J. Dairy Sci., 46: 396. (1963).

88. PATTON, S., and McCARTHY, R.D.
Structure and synthesis of milk fat. V. A postulated sequence of events from analyses of mammary tissue lipids.
J. Dairy Sci., 46: 916. (1963).

89. PHATAK, S.S., and PATWARDHAN, V.N.
Iso-oleic acids in cow and buffalo milk fat.
Nature, 172: 456. (1953).

90. POPJAK, G., FRENCH, T.H., and FOLLEY, S.J.
Utilization of acetate for milk fat synthesis in the lactating goat.
Biochem. J., 48: 411. (1951).

91. POPJAK, G., FRENCH, T.H., HUNTER, G.D., and MARTIN, A.J.P.
Mode of formation of milk fatty acids from acetate in the goat.
Biochem. J., 48: 612. (1951).

92. RIIS, P.M., LUICK, J.R., and KLEIBER, M.
Role of plasma lipids in transport of fatty acids for butterfat formation.
Am. J. Physiol., 198: 45. (1960).

93. SAMBASIVARAO, K., and BROWN, J.B.
The nature of the C_{18} Polyethenoic fatty acids of butter fat.
J. Am. Oil Chem. Soc., 39: 340. (1962).

94. SCHOLFIELD, C.R., and HICKS, M.A.
Glyceride structure of vegetable oils by counter-current distribution. II. Soybean oil.
J. Am. Oil Chem. Soc., 34: 77. (1957).

95. SCHOLFIELD, C.R., and DUTTON, H.J.
Glyceride structure of vegetable oils by counter-current distribution. III. Safflower oil.
J. Am. Oil Chem. Soc., 35: 493. (1958).

96. SCHOLFIELD, C.R., NOWAKOWSKA, J., and DUTTON, H.J.
Glyceride structure of vegetable oils by counter-current distribution. VI. Corn oil.
J. Am. Oil Chem. Soc., 38: 175. (1961).

97. SCHWENK, E., and WERTHESSEN, N.T.
Studies on the biosynthesis of cholesterol.
III. Purification of C^{14}-cholesterol from perfusions of livers and other organs.
Archs Biochem. Biophys., 40: 334. (1952).

98. SCOTT, W.E., HERB, S.F., MAGIDMAN, P., and RIEMENSCHNEIDER, R.W.
Unsaturated fatty acids of butterfat.
J. agric. Fd Chem., 7: 125. (1959).

99. SHORLAND, F.B.
C_{18} unsaturated acids of butterfat.
Nature, 166: 745. (1950).

100. SHORLAND, F.B., GERSON, T., and HANSEN, R.P.
The branched-chain fatty acids of butterfat.
6. Further investigations of the C_{15} saturated acids.
Biochem. J., 59: 350. (1955).

101. SHORLAND, F.B., GERSON, T., and HANSEN, R.P.
Branched-chain fatty acids of butterfat.
7. Investigation of the C_{13} acids.
Biochem. J., 61: 702. (1955).

102. SMEDLEY, I.
The fatty acids of butter.
Biochem. J., 6: 451. (1912).

103. SMITH, L.M., FREEMAN, N.K., and JACK, E.L.
The unsaturated fatty acids of milk fat.
III. Geometrical isomerism.
J. Dairy Sci., 37: 399. (1954).

104. SMITH, L.M., FREEMAN, C.P., and JACK, E.L.
Distribution of fatty acids in milk fat fractions.
J. Dairy Sci., 48: 531. (1965).

105. SUBBARAM, M.R., and YOUNGS, C.G.
Determination of the glyceride structure of fats: Distribution of individual saturated and unsaturated acids.
J. Am. Oil Chem. Soc., 41: 445. (1964).

106. van den TEMPEL, M., de BRUYNE, P., and MANK, A.P.J.
Crystallization chromatography of glycerides.
Recl Trav. chim. Pays-Bas, 81: 1075. (1962).

107. WINDAUS, A.
Uber die entgiftung der saponine durch cholesterin.
Ber. 42: 238. (1909).
Cited by Cook, R.P. Cholesterol.
Academic Press Inc., New York. (1958).

108. WHITE, M.F., and BROWN, J.B.
A study of the tetrabromide method of estimating linolenic acid in fatty acid mixtures.
J. Am. Oil Chem. Soc., 26: 385. (1949).